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Determination of Plasma Soluble Fibrin Using a New ELISA Method in Patients With Disseminated Intravascular Coagulation

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We measured plasma levels of soluble fibrin (SF) in 98 patients suspected of having disseminated intravascular coagulation (DIC) using a newly developed enzyme-linked immunosorbent assay (ELISA) and investigated the correlations between SF determinations and measurements of other hemostatic molecular markers to determine the diagnostic usefulness of determinations of SF. Patients were classified into four groups according to their clinical and laboratory findings: overt DIC (n = 33), subclinical DIC (n = 23), hypercoagulability (n = 22), and non-DIC (n = 20). SF levels were significantly higher in patients with overt DIC compared with the other three groups and were significantly higher in the subclinical DIC and hypercoagulability groups compared with the non-DIC patients. SF levels increased significantly with each increase in the clinical stage. Although levels of thrombin-antithrombin III complex (TAT), prothrombin fragment 1 + 2 (PF_{1+2}), cross-linked fibrin degradation products (XDP), and plasmin-antiplasmin complex (PAP) were significantly increased in patients with overt DIC compared with non-DIC patients, the values of these hemostatic molecular markers did not consistently show an increase in association with advances in the disease stage. Plasma levels of SF in patients with overt DIC showed a positive correlation with levels of TAT, XDP, and FDP(E), but not with PF_{1+2} and PAP. Analysis of receiver-operating characteristic curves showed that the sensitivity and specificity of SF were similar to those of XDP for diagnosis of DIC. The sensitivity and specificity of SF for diagnosis of overt DIC were both above 90% when the cut-off value was set at 65 μg/ml. Plasma levels of SF were also increased in patients with extravascular fibrin formation without DIC.

Our findings suggest that measurement of plasma levels of SF by this ELISA method is useful for the diagnosis of DIC and the evaluation of the patient's clinical status. © 1996 Wiley-Liss, Inc.

Key words: soluble fibrin, disseminated intravascular coagulation, hemostatic molecular markers, hypercoagulable state

INTRODUCTION

Disseminated intravascular coagulation (DIC) is associated with various disease states. Thus, the pathophysiology of DIC is diverse and its clinical manifestations depend on the nature of the underlying disorder [1]. Systemic intravascular microthrombus formation seems to be a common pathophysiologic finding in DIC. Thus, the early detection of microthrombus formation may be useful in diagnosing DIC before the full-blown state develops, when there is an increased incidence of fatal bleeding or multiple organ failure.

Assays of highly sensitive and specific molecular makers of microthrombus formation have been developed for laboratory use [2]. Previous methods for the detection of thrombin generation have been directed at measuring the levels of zymogens, inhibitors, or substrates in the coagu-

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lation cascade. These molecular species are present in excessive amounts in blood and show only a small decrease in vivo. Measurement of substances that appear or increase significantly during the generation of thrombin or plasmin including enzyme-inhibitor complexes, such as thrombin-antithrombin III complex (TAT) and plasmin-antiplasmin complex (PAP), and by products, such as fibrinopeptide A and prothrombin fragment 1 + 2 (PF_{1+2}) and products, such as fibrin monomers, cross-linked fibrin degradation products (XDP), and fibrin and fibrinogen degradation products (FDP), of the enzyme reactions of coagulation and fibrinolysis, are useful for detection of a hypercoagulable state [2]. However, molecular markers such as TAT and PF_{1+2} reflect intravascular thrombin generation, but do not directly reflect microthrombus formation. Thrombin generation is regulated by several important anticoagulant systems, including inhibition by antithrombin III-glycosaminoglycan complex and binding of thrombomodulin, fibrinogen, and endothelial thrombin receptor protein [3]. Thus, in the presence of systems that inhibit thrombin, thrombin generation does not always result in microthrombus formation. Plasma levels of soluble fibrin (SF) may accurately reflect the fibrinogen-converting activity of thrombin. However, several assays for SF so far described give only qualitative results [4–6].

In the present study, we investigated the diagnostic usefulness of determination of the plasma level of SF using a newly developed ELISA method in patients with DIC. We compared the usefulness of SF determination with measurements of other hemostatic molecular markers of thrombin and plasmin generation using receiver-operating characteristic curves.

PATIENTS AND METHODS

We studied 98 patients suspected of having DIC. Underlying disorders included carcinoma (n = 31), leukemia (n = 16), sepsis (n = 15), collagen disease (n = 9), hepatic failure (n = 7), post-operation (n= 6), aortic aneurysm (n = 2), cardiac failure (n = 2), cerebral vascular infarction (n= 2), and miscellaneous diseases (n= 8).

DIC was diagnosed on the basis of detection of soluble fibrin monomer complex (SFMC) and the determinations of FDP (E) levels as previously described [7]. These two molecular markers are believed to directly reflect formation of microthrombi [8]. Furthermore, these two tests are easy to perform and give the quick answers, which can be useful for the early diagnosis of DIC. Thus, we made the following criteria for a diagnosis of DIC: 1) presence of underlying disorders frequently associated with DIC, 2) positive SFMC and elevated FDP(E) levels (higher than 500 ng/ml), and/or 3) presence of clinical bleeding or organ dysfunction. The patients were classified into four groups based on the clinical status and the test results: overt DIC (criteria 1 + 2 + 3), subclinical DIC (criteria 1 + 2), hypercoagulability (criteria 1 + positive SFMC), and non-DIC (criteria 1). Patients with subclinical DIC and hypercoagulability do not always develop overt DIC. Thus, the pathologic features of such patients did not always represent preliminary stage of overt DIC, but were associated with the patients' various underlying disorders.

We also studied 16 patients with extravascular fibrin formation without DIC (n = 16) who exhibited gastrointestinal bleeding (n = 10) or ascites (n= 6). These patients did not meet the criteria for DIC or hypercoagulability.

Plasma levels of TAT [9] and PF_{1+2} [10] were determined using previously described ELISA methods. Levels of XDP and FDP (E) were determined by the latex agglutination method using commercially available kits (Iatron Co., Tokyo, Japan) [11]. The PAP level was determined by a one-step sandwich enzyme immunoassay using polyclonal anti-plasminogen antibody-coated polystyrene balls and a peroxidase-conjugated monoclonal anti-antiplasmin antibody (Teijin Co., Tokyo, Japan) [12]. SFMC was determined by a red cell agglutination method [6]. SF levels were determined by a newly developed ELISA method that uses a fibrin specific monoclonal antibody (2B5) raised against the synthetic N-terminal heptapeptide (Gly-Pro-Arg-Val-Val-Glu-Arg) of the fibrin alpha-chain [13]. The epitope structure is exposed after removal of fibrinopeptide A from fibrinogen by the action of thrombin. The 2B5 monoclonal antibody was used both as a biotinylated capture antibody and as a peroxidase-labelled antibody. This ELISA is a two-step sandwich assay using streptavidin-coated tubes as solid phase. Plasma samples were preincubated with KSCN to prevent complex formation between fibrin monomers and fibrinogen. Human plasma spiked with high molecular weight fibrin was supplied by the manufacturer and was used as a calibrator. Detectable molecular species of fibrin molecules detected by this ELISA system are fibrin monomers and the X, Y, and E fragments of fibrin molecules [13,14]. The normal plasma level of SF (the 95th percentile), as determined by measurement in 116 healthy volunteers (56 men and 60 women) is <6.6 μg/ml.

Receiver-operating characteristic (ROC) curves were generated by plotting sensitivity (true-positive rate) vs. 1-specificity (false-positive rate). Sensitivity (true positives/ [true positives + false negatives]) and specificity (true negatives/ [true negatives + false positives]) were calculated over the entire range of the analyte. The area under the ROC curves was calculated according to a previously described method [15]. Areas under the ROC curves were compared using the method described by Hanley and McNeil [16]. Differences between groups were analyzed by the Student's t-test. A P value of <0.05 indicated statistical significance.

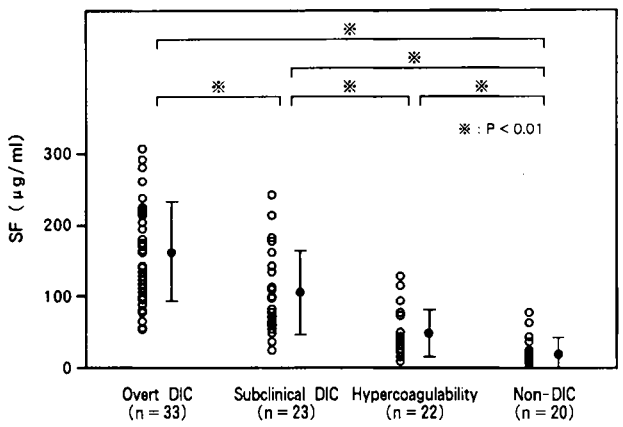

Fig. 1. Plasma levels of soluble fibrin (SF) in patients with overt DIC, subclinical DIC, hypercoagulability and non-DIC. Plasma levels of SF were measured by an ELISA method as described in the Method section. Definition of overt DIC, subclinical DIC, hypercoagulability, and non-DIC were described in Patients and Methods. Data are expressed as mean ± SD. Statistical significance was analyzed using Student's t-test. *P*-value is shown in the figure.

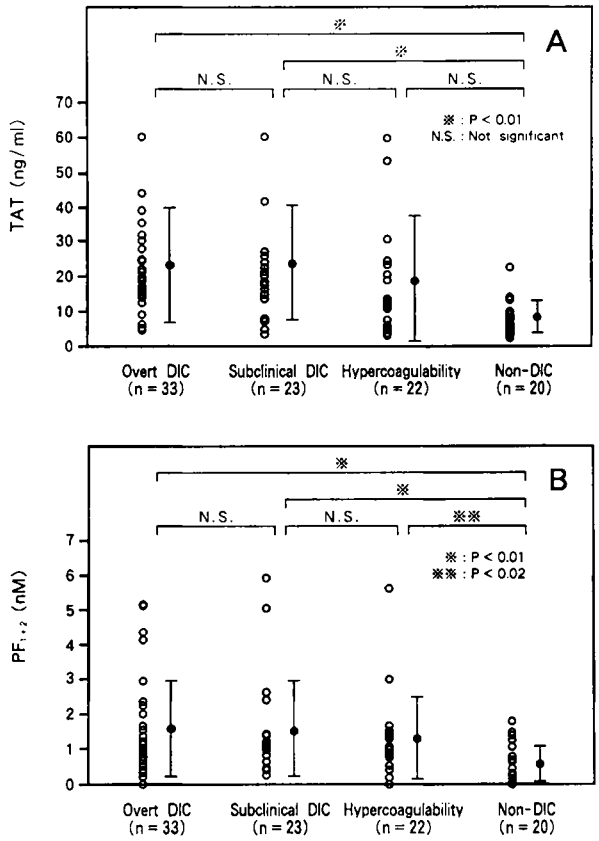

Fig. 2. Plasma levels of thrombin-antithrombin III complex (TAT) (A) and prothrombin fragment 1 + 2 (PF_{1+2})(B) in patients with overt DIC, subclinical DIC, hypercoagulability and non-DIC. Plasma levels of TAT and PF_{1+2} were measured by ELISA method as described in [9] and [10], respectively. Details as for Figure 1. Data are expressed as mean ±SD. *P*-values are shown in the figure.

RESULTS

Plasma SF Levels in 98 Patients Suspected of Having DIC

Plasma levels of SF were determined in 98 patients suspected of having DIC. Overt DIC was identified in 33 patients, subclinical DIC in 23 patients, hypercoagulability in 22 patients, and non-DIC in 20 patients. The SF levels increased significantly with each increase in the clinical stage (Fig. 1). Plasma levels of TAT, PF_{1+2}, XDP, FDP(E) and PAP were significantly higher in patients with DIC compared with non-DIC patients (Fig. 2, 3). However, these assay values did not show consistent increases with each increase in clinical stage. The plasma level of SF in patients with overt DIC was significantly correlated with the values for TAT (correlation coefficient, $r = 0.54$), $P < 0.02$), XDP ($r = 0.51$, $P < 0.01$), and FDP(E) ($r = 0.54$, $P < 0.01$) (Table I).

These findings suggest that plasma SF levels increase in pathologic conditions of DIC with the advance of the clinical stages, and that the increase of SF may be deeply related to the activation of coagulation and fibrinolysis.

ROC Curve Analysis

Analysis of ROC curves showed that the sensitivity and specificity of the SF level for diagnosis of overt DIC were similar to the sensitivity and specificity of XDP (Fig. 4A). For diagnosis of subclinical DIC, the sensitivity and specificity of the XDP determination were highest of the five factors studied (Fig. 4B). The sensitivity and specificity of the SF determination appeared to be highest for diagnosis of hypercoagulability (Fig. 4C). The areas under the ROC curves were similar for SF and XDP, and these values were higher than those of other molecular markers tested in the present study (Table I).

Sensitivity and Specificity of SF for Diagnosis of DIC

Sensitivity and specificity of SF were calculated for each group of patients suspected of having DIC. The sensitivity and specificity of SF for overt DIC were 93.9% and 95.0%, respectively, when the cut-off value was set at 65 μg/ml. SF had a sensitivity of 91.3% and a specificity of 90% for subclinical DIC when the cut-off value was set at 50 μg/ml. The sensitivity and specificity of XDP for both overt DIC and subclinical DIC were >90%. SF had a sensitivity of 81.3% and a specificity of 80.0% for hypercoagulability when the cut-off value was set at 20 μg/ml.

These findings suggest that SF determination by the ELISA method can be of diagnostic value for DIC.

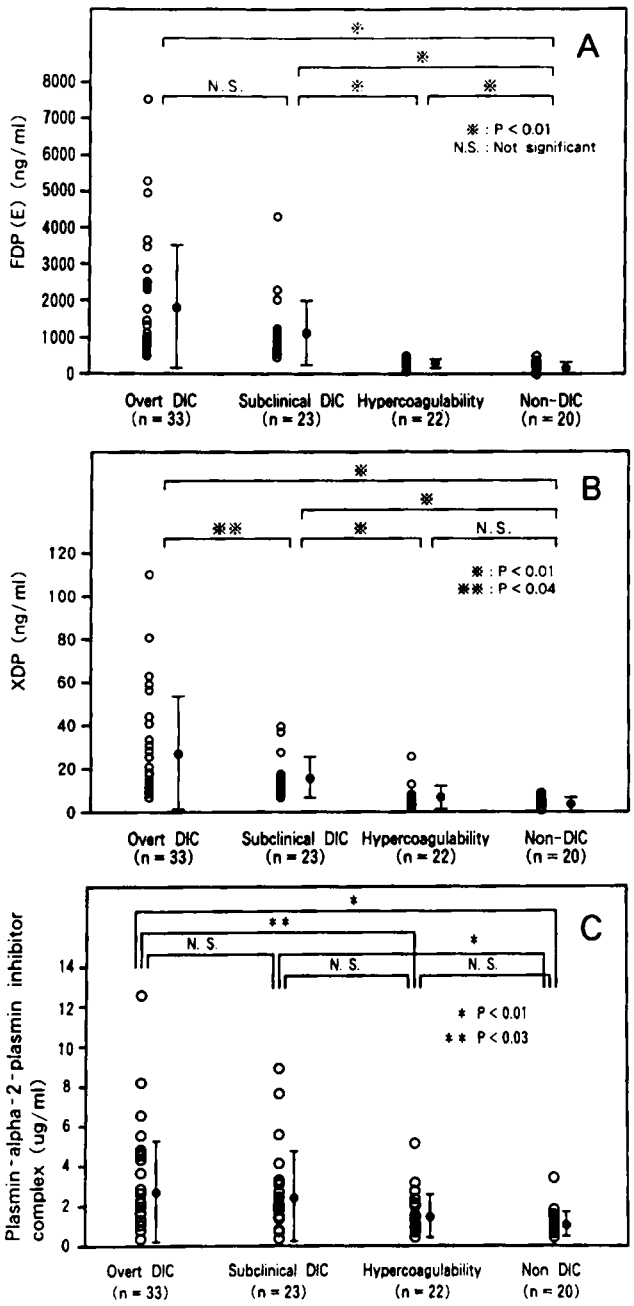

Fig. 3. Plasma levels of fibrin and fibrinogen degradation products (E) [FDP(E)] (A), cross-linked fibrin degradation products (XDP) (B), and plasmin-antiplasmin complex (PAP) (C) in patients with overt DIC, subclinical DIC, hypercoagulability and non-DIC. Plasma levels of FDP(E) and XDP were measured by a latex agglutination method as described in [2]. PAP levels were measured by a ELISA method as previously described [12]. Details as for Figure 1. Data are expressed as mean ± SD. *P*-values are shown in the figure.

Plasma Levels of SF in Patients With Extravascular Fibrin Formation

Plasma levels of SF, XDP, and TAT in patients with extravascular fibrin formation without DIC were significantly lower than in patients with overt DIC and significantly higher than in non-DIC patients (Table II). The plasma level of PF_{1+2} was similar in patients with extravascular fibrin formation and patients with overt DIC. The plasma level of PAP was significantly lower in patients with extravascular fibrin formation than in patients with overt DIC, but was not increased significantly compared with non-DIC patients.

TABLE I. AUCs of Various Molecular Markers of Coagulation and Fibrinolysis[a]

Markers	AUC		
	Overt DIC	Subclinical DIC	Hypercoagulability
SF	0.992	0.963	0.831
XDP	0.999	0.998	0.830
TAT	0.880	0.893	0.768
PF_{1+2}	0.783	0.800	0.727
PAP	0.839	0.770	0.668

[a]AUC, area under the receiver-operating characteristic curve; SF, soluble fibrin; XDP, cross-linked fibrin degradation products; TAT, thrombin-antithrombin III complex; PF_{1+2}, prothrombin fragment 1 + 2; PAP, plasmin-antiplasmin complex.

DISCUSSION

Our results showed that SF was a useful molecular marker of coagulation abnormalities and that the SF level reflected the clinical stage of patients suspected of having DIC. Analysis of ROC curves showed that ELISA determinations of SF were of diagnostic value for DIC. SF may accurately reflect microthrombus formation, which may be a key factor in the clinical manifestations of DIC [17].

Measurement of SFMC by the red cell agglutination method is simple and quick, but it gives only qualitative results [6]. In a preliminary study, the red cell agglutination method failed to detect SFMC during the 7 days preceding onset of overt DIC in patients (n = 22), whereas the plasma level of SF was higher in these patients than in non-DIC patients (n = 29) who did not develop DIC within a week. The usefulness of SF determinations for preceding the onset of DIC at an early phase needs to be examined in a well-controlled prospective study.

SF, which directly reflects microthrombus formation, was a useful diagnostic marker of overt DIC, subclinical DIC and hypercoagulability in the present study. However, plasma SF can be derived not only from intravascular fibrin, but also from extravascular fibrin [18]. In the present study, plasma levels of SF and XDP were increased in patients with extravascular fibrin formation without DIC (the patients with gastrointestinal bleeding or ascites). Thus, it is possible that plasma levels of SF and XDP may reflect the formation of both intravascular and extravascular fibrin, suggesting that the results of

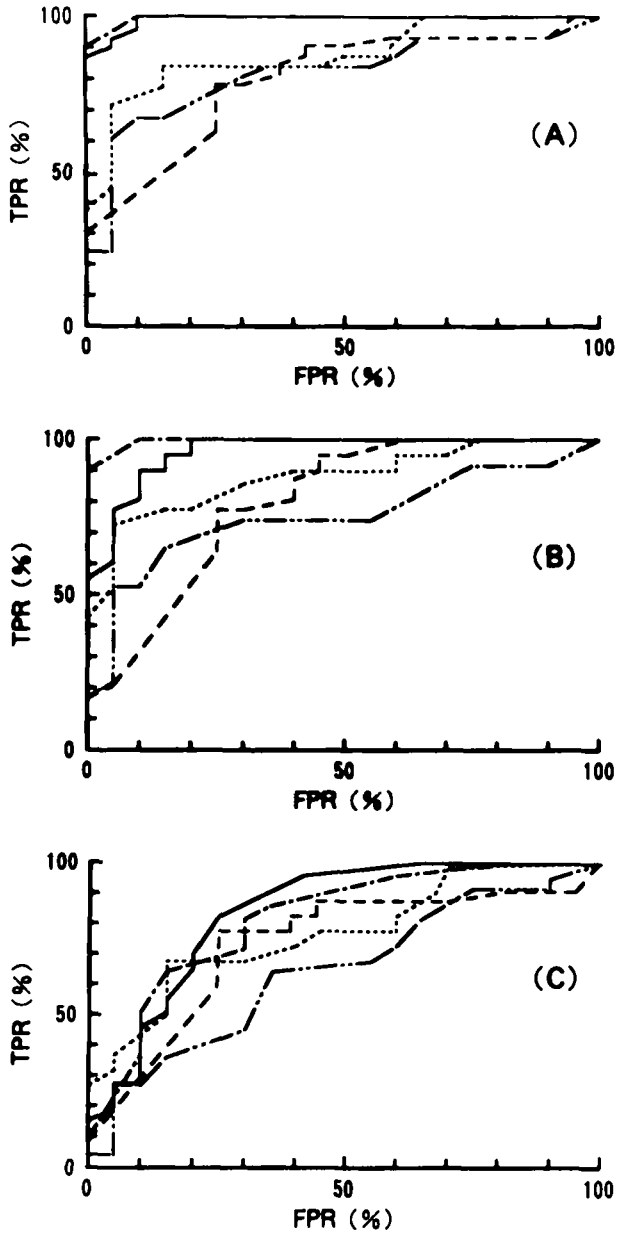

Fig. 4. Receiver operating characteristic curve of diagnostic molecular markers for overt DIC (A), subclinical DIC (B), and hypercoagulability (C). Receiver operating characteristic curve generation of fibrin monomers and the other hemostatic molecular markers was performed in patients with overt DIC (A), subclinical DIC (B), and non-DIC (C). TPR, true-positive rate; FPR, false-positive rate. Soluble fibrin, ——; thrombin-antithrombin III complex, ------; prothrombin fragment 1 + 2, – – –; cross-linked fibrin degradation products, -------; plasmin-antiplasmin complex, —·—·—.

assays of SF and XDP may be difficult to evaluate in patients with gastrointestinal bleeding or ascites.

Assays of SF and XDP appear to have a similar usefulness for the diagnosis of DIC. However, the cost of SF determinations performed with an automated device (ES-300, Boehringer Mannheim Co., Mannheim, Germany) is about two thirds of the costs of XDP determinations (LPIA-100, Iatron Co. Tokyo, Japan) and the assay can be completed in 2.5 min/test compared with 10 min/test for the assay of XDP.

TABLE II. Plasma Levels of SF and Other Molecular Markers in Patients With Extravascular Fibrin Formation[a]

		Overt DIC (n = 33)	Non-DIC (n = 20)	Extravascular fibrin formation (n = 16)
SF	(μg/ml)	165.2 ± 68.8	19.3 ± 20.0*	115.2 ± 62.0§†
XDP	(μg/ml)	26.8 ± 24.7	2.5 ± 1.7*	9.1 ± 4.3*†
TAT	(ng/ml)	23.5 ± 16.0	6.8 ± 4.7*	10.1 ± 9.3*†
$PF_{1,2}$	(nM)	1.62 ± 1.36	0.58 ± 0.55*	1.97 ± 0.78†
PAP	(μg/ml)	2.71 ± 2.52	0.96 ± 0.63*	1.21 ± 0.82¶

[a]SF, soluble fibrin, XDP, cross-linked fibrin degradation products; TAT, thrombin-antithrombin III complex; PF_{1+2}, prothrombin fragment 1 + 2; PAP, plasmin-antiplasmin complex.
*$P < 0.01$ vs. Overt DIC; §$P < 0.02$ vs. Overt DIC; ¶$P < 0.03$ vs. Overt DIC; †$P < 0.01$ vs. Non-DIC.

Plasma levels of plasminogen activator inhibitor-1 (PAI-1) and α_2-plasmin inhibitor are increased in the acute phase in patients with DIC associated with sepsis [19,20], suggesting that fibrinolysis is decreased in patients with sepsis. Because generation of XDP is dependent on fibrinolytic activity in patients with DIC, plasma levels of XDP may not increase in patients with sepsis, despite systemic formation of microthrombi. Further study is needed to examine the correlations between plasma levels of SF or XDP and the clinical manifestations of DIC in patients with sepsis to determine which marker is more useful for the diagnosis of DIC in association with sepsis.

REFERENCES

1. Muller-Berghaus G: Pathophysiologic and Biochemical Events in Disseminated Intravascular Coagulation: Dysregulation and Anticoagulant Pathways. Semin Thromb Haemost 15:58, 1989.
2. Bauer KA, Rosenberg RD: The pathophysiology of the prethrombotic state in humans: Insights gained from studies using markers of hemostatic activation. Blood 70:343, 1987.
3. Bone RC: Modulators of coagulation. Arch Intern Med 152:1381, 1992.
4. Godal HC, Abildgaard U: Gelation of soluble fibrin in plasma by ethanol. Scand J Hematol 3:324, 1966.
5. Niewiarowski S, Gurewich V: Laboratory identification of intravascular coagulation: The serial dilution protamine sulfate test for the detection of fibrin monomer and fibrin degradation products. J Lab Clin Med 77:665, 1971.
6. Largo R, Heller V, Straub W: Detection of soluble intermediate for the fibrinogen-fibrin monomers. Blood 47:991, 1976.
7. Okajima K, Fujise R, Motosato Y, Ushijima M, Okabe H, Takatsuki K: Plasma levels of granulocyte elastase-1-proteinase inhibitor complex in patients with disseminated intravascular coagulation: Pathophysiologic implications. Am J Hematol 47:82, 1994.
8. Spero JA, Lewis JH, Hashiba U: Disseminated intravascular coagulation: Findings in 346 patients. Thromb Haemost 43:28, 1980.
9. Pelzer H, Schwarz A, Heimburger N: Determination of human throm-

bin-antithrombin III complex in plasma with an enzyme-linked immunosorbent assay. Thromb Haemost 59:101, 1988.
10. Pelzer H, Schwarz A, Heimburger N: Determination of human prothrombin activation fragment 1 + 2 in plasma with an antibody against a synthetic peptide. Thromb Haemost 65:153, 1988.
11. Okajima K, Kohno I, Soe G, Okabe H, Takatsuki K, Binder BR: Direct evidence for systemic fibrinogenolysis in patients with acquired alpha-2-plasmin inhibitor deficiency. Am J Hematol 45:10, 1994.
12. Mimuro J, Koike Y, Sumi Y, Aoki N: Monoclonal antibodies to discrete regions in alpha-2-plasmin inhibitor. Blood 69:446, 1987.
13. Lill H, Spannagl M, Trauner A, Schramm W, Schuller D, Ofenloch-Haehnle B, Draeger B, Naser W, Dessauer A: A new immunoassay for soluble fibrin enables a more sensitive detection of the activation state of blood coagulation in vitro. Blood Coag Fibr 4:97, 1993.
14. Dempfle CE, Dollman M, Lill H, Puzzoivo D, Heene DL: Binding of a new monoclonal antibody against N-terminal heptapeptide of fibrin alpha-chain to polymerization site "A": Effects of fibrinogen and fibrinogen derivatives, and pretreatment of sample with NaSCN. Blood Coag Fibr 4:79, 1993.
15. Cembrowski GS, Griffin JH, Mosher DF: Diagnostic efficacy of six plasma proteins in evaluating consumptive coagulopathies. Arch Intern Med 146:1997, 1986.
16. Hanley JA, McNeil BJ: A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology 148:839, 1983.
17. Nieuwenhuizen W: Soluble fibrin as a molecular marker for a prethrombotic state: a mini-review. Blood Coag Fibr 4:93, 1993.
18. Dvorak HF, Senger DR, Dvorak AM: Fibrin as a component of the tumor stroma: Origins and biological significance. Cancer Metastasis Rev 2:41, 1983.
19. Kruitof EKO, Gundinchet A, Bachman F: Plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 in various disease states. Thromb Haemost 59:7, 1988.
20. Matsuda M, Wakabayashi K, Aoki, N: α_2-Antiplasmin inhibitor is among acute phase reactants. Thromb Res 17:527, 1980.